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PROBLEM OF SINGLE CELL VERSUS MULTICELL ORIGIN OF A TUMOR

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1. Introduction

Cell markers have been used to study the cell population which gives rise to tumors. These traits are presumed to be transmitted to daughter cells and so set apart a cell or group of cells from surrounding cells of like type. Chromosome markers have been used to study experimentally induced and naturally occurring tumors [1], [8]. Such markers are usually considered chance findings, primarily serving to follow the growth of a particular cell line. The chromosomal variant itself may also be involved in tumor formation as strongly suggested by finding a consistently abnormal chromosome, the Philadelphia chromosome, in the leukocytes of individuals with chronic myelogenous leukemia [14]. Antigenic specificity of cells has also been employed to differentiate otherwise similar cells within a tumor [10], [16].

We have used a fixed and natural cell marker, the electrophoretic variant of glucose-6-phosphate dehydrogenase (G6PD) to study the cell population of leiomyomas of the uterus. G6PD is an enzyme whose gene locus in man lies on the X chromosome. Females heterozygous for G6PD have two cell populations each expressing one of the two alleles, that is to say, an individual heterozygous for G6PD has a mixture of cells some of which show one or the other but not both characteristics. The two cell populations breed true throughout somatic cell growth. This is presumed to be due to random inactivation of all or part of one of the two X chromosomes early in development. The inactive X chromosome remains inactive in all daughter cells and prevents the phenotypic expression of the G6PD locus on that particular X chromosome [2], [4], [13], [17].

If tumors from individuals heterozygous for G6PD arise from single cells, they should have a single phenotype and with this idea in mind, we have studied normal tissues and tumors from such people [7], [11], [12]. Several genetic variants of this enzyme are known. Some of these are quantitative variants while others are variants demonstrable by electrophoresis [3], [9]. We chose the

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electrophoretic variant of G6PD seen in Negroes because individuals with this variant are common and it is relatively easy to separate heterozygous from homozygous individuals. The particular variant used in this study has a gene frequency of about 0.34 in Negroes in the United States. The variant (A) migrates about 10 per cent faster on starch gel electrophoresis than the non-Negro (B) enzyme. There are two genotypes among individuals with the (A) band. In one (Gd^{A-}) the red blood cells and, to a lesser extent, other tissues have a decreased G6PD activity while the genotype (Gd^{A+}) has normal enzyme activity [5].

We have studied leiomyomas of the uterus from females heterozygous for the electrophoretic variant of G6PD. Leiomyomas are tumors made up of smooth muscle fibers. They are discrete, easy to diagnose on gross examination, available for biochemical analysis and usually multiple. Although they appear encapsulated, they are not; the periphery of these tumors merges with the adjacent myometrium and is discarded prior to the analysis of the tumor itself. An estimated 90 per cent or more of the tumor mass is composed of smooth muscle cells. The rest consists of blood vessels, fibrocytes and histiocytes of various types such as mast cell, lymphocytes, and macrophages. The myometrium has greater vascularity than the leiomyomas.

The uteri used for the study were obtained from individuals undergoing routine surgical procedures in San Francisco and Seattle. One and frequently two or more segments approximately 0.6 cm^3 were analyzed to determine the electrophoretic type, fast (A), slow (B) or both fast and slow (AB). Except in two cases, no attempt was made to determine the Gd^{A+} and Gd^{A-} phenotype among those individuals with a fast electrophoretic band.

The myometrial and tumor samples were homogenized, subjected to vertical starch gel electrophoresis and stained for G6PD as previously described [11]. The relative amounts of A and B cells (expressed as $A:B$) were distinguished from no detectable A (85 to 95 per cent B) to no detectable B (85 to 95 per cent A). The standards for classification were established from known mixtures of A and B cells. The sensitivity varied from time to time. About twenty leiomyomas were examined for a sex chromatin body. In all cases, there was a single chromatin body, an indication of two X chromosomes per cell.

2. Results

Seventeen uteri from heterozygous females with one or more leiomyomas were studied (table I). In this series 206 segments of myometrium ranging from 1 to 10 mm^3 were examined and all but one segment had both A and B bands present. At least three separate segments from different parts of myometrium were examined in each case and in many of them 1 mm^3 segments were obtained from a single segment of myometrium and analyzed. Here too both A and B bands were found in all samples. The presence of a single band in the myometrium of a heterozygous individual is interesting and we have seen a single band

TABLE I

G6PD ELECTROPHORETIC STUDIES OF MYOMETRIUM AND LEIOMYOMAS FROM *AB* HETEROZYGOTES

tr indicates trace amount.

	Myometrium Phenotype				Tumor Phenotype								
					Normal Tumors without Capsules			Normal Tumors with Capsules			Necrotic Tumors without Capsules		
Case	*A*	*B*	*AB*	Average *A*:*B* Ratio	*A*	*B*	*AB*	*A*	*B*	*AB*	*A*	*B*	*AB*
134	0	0	3	2:3	1	0	0						
139	0	0	4	2:3	0	0	0			1(tr:4)			
3	0	0	7	—	2	3	0						
4	0	1	6	—	4	1	0						
5	0	0	14	—	5	2	0						
6	0	0	18	—	3	1	0						
11	0	0	40	1:1	3	3	0						
40	0	0	4	1:1	1	0	0			1(3:1)		1	2(1:1) (3:tr)
41	0	0	3	1:3	0	1	1(3:1)						
43	0	0	19	1:2								1	
49	0	0	13	3:2	10	3	0						
51	0	0	13	3:2	7	3	0						
60	0	0	4	2:3	1	4	0						1(2:3)
67	0	0	13	3:2	3	5	0						
S6	0	0	30	3:1	5	1	0						
S16	0	0	3	1:4	4	23	0						
73	0	0	11	1:3	4	18	0						
Total	0	1	205		53	68	1						

in an otherwise heterozygous individual in a 100 mm^2 piece of cervical epithelium and red blood cells in case 73.

The findings in the uterus are in sharp contrast to those of the leiomyomas. These can be summarized as follows.

(1) The number of leiomyomas examined was 129; of these 122 had the usual whitish color of a viable tumor and were large enough to separate from their capsules. These ranged from 4 to 60 mm in maximum diameter. In this group of tumors the phenotype, with one exception, was all *A* or all *B* (figure 1). Both *A* and *B* tumors were found in the same uterus containing multiple leiomyomas. The exception was a leiomyoma which had a weak second band (case 41). It had been removed with an early pregnancy. The two leiomyomas analyzed with their periphery were the smallest tumors used in this study, one measuring 0.3 and the other 0.4 cm in maximum diameter. Both had a weak second band. Among the five tumors which were grossly necrotic, there were three which had a double band.

Microscopic sections of the myoma within a pregnant uterus and two of the necrotic leiomyomas containing a double band revealed a prominent vascular component which we feel accounts for a second band within these tumors. Since

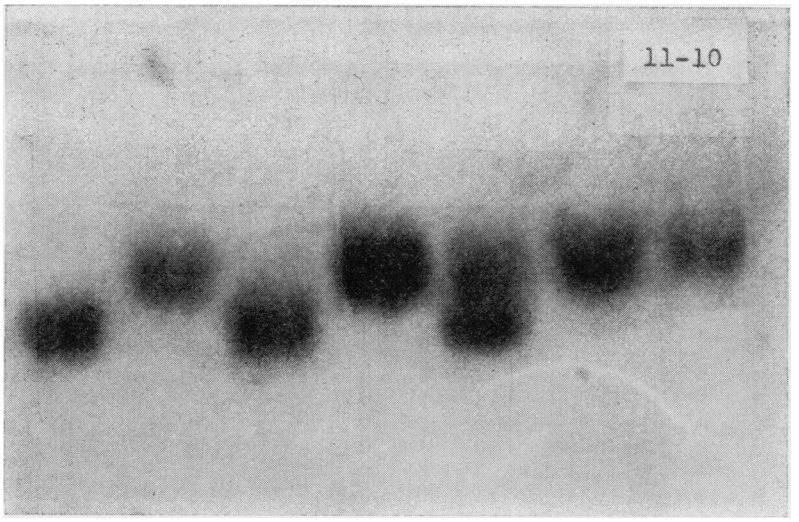

FIGURE 1

G6PD electrophoretic analysis of multiple leiomyomas and uterus from case 51. The *A B C D F G* are separate leiomyomas showing an *A* or *B* G6PD band contrasted to the myometrium which contains both *A* and *B* bands (*E*).

the capsular tissue has been shown to contain adherent myometrium we are inclined to believe contamination with the myometrium accounts for the weak second band within the two small tumors.

(2) The total number of *A* and of *B* tumors is about equal but the variation in the number of *A* and *B* tumors in any particular uterus is great. Some uteri contain mostly *A* tumors while *B* tumors predominate in others. This is shown graphically in figure 2. A chi square analysis was performed on the deviation from an expected mean of 1:1 of *A* and *B* tumors in each uterus and the likelihood that our deviation from 1:1 is due to chance is less than 0.1 per cent.

(3) Except for case 67 the predominant cell type in the myometrium of the 12 cases containing multiple tumors was the same as the predominant type seen in the tumors within that uterus. The variation of the *A*:*B* ratio in the myometrium was not as great as the variation of *A* and *B* tumors (figure 2).

3. Discussion

The finding that the leiomyomas of the uterus were of a single phenotype as contrasted to the myometrium in which small samples had both *A* and *B* cells represented is consistent with the concept that these tumors arose from single cells.

Our data, however, does not rule out the possibility that these tumors arose from several cells but that only one phenotype was present at the time of anal-

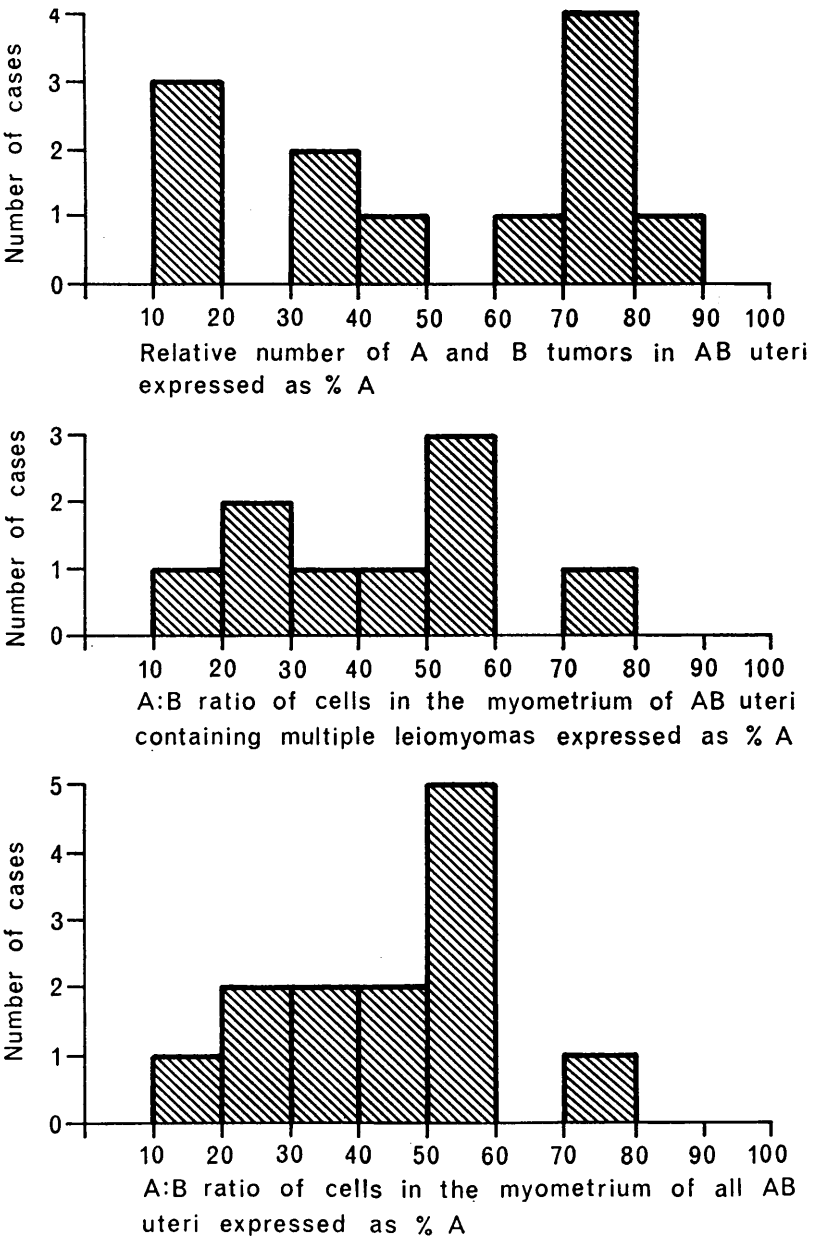

FIGURE 2

Distribution of *A* and *B* tumors in separate uteri heterozygous for 6-6PD (upper graph).
Relative number of *A* and *B* cells in separate uteri heterozygous for G6PD (middle and lower graph).

ysis. This could have come about if the cells giving rise to the tumor were of like phenotype or were of a different phenotype but one phenotype emerged due to selection. A minor second band in two small tumors has been interpreted as representing cells other than those of the tumor but it could also indicate that these tumors and possibly the others as well arose from several cells but only those cells with a single phenotype persisted during subsequent growth. Selective forces could lie on the X chromosome or could be a single event such as a mutation within one of the several cells making up the original neoplasm. They could lie within the genotype of the cells or be an end result of random life and death processes acting upon a small population of cells resulting in the outgrowth of a single cell. The possible modifying factors to be discussed suggest that selective forces are influencing the growth of the tumors and/or the myometrium, but the presence of a single phenotype within all of the larger tumors is very strong evidence that they arose from single cells. In keeping with this idea, at least thirty 1 mm^3 samples taken from the core of two of the larger tumors failed to show residual tumor cells of another phenotype.

Active growth does not necessarily cause selection of one cell type over the other. This can be seen when comparing the $A:B$ ratio of the uterine endometrium with that of the myometrium. In one case not included in this study and in case 73 the endometrium has the same $A:B$ ratio as the myometrium. Since the endometrium sloughs with each monthly menstrual cycle, while the myometrium normally has very little (if any) cell turnover, it is unlikely that active growth *per se* causes selection.

Cells of like G6PD phenotype could give rise to these tumors if the patches of pure A and pure B cells were large. If A and B cells were simply distributed at random within the myometrium they would form small aggregates of like type in continuity with one another (patch). Larger patches would be formed if daughter cells remained next to one another to form a coherent clone of cells. If there was extensive migration of daughter cells, then groups of like cells would be small and irregular in shape and the chance that adjacent cells will be alike is relatively low. With clonal growth, patches of like cells would be relatively large and the chance that adjacent cells will be alike would be large. (Patches similar to coherent clonal growth could also arise if cells of like type attracted one another.)

We have attempted to determine the patch size of pure A and pure B cells within the myometrium. Since A and B cells were seen in the smallest samples which we could analyze (1 mm in maximum diameter) the patches must be considerably smaller than the sample size. We have estimated their number.

This estimate is based upon the fact that the degree of heterogeneity is inversely proportional to the number of patches within each sample. Sample measurements were converted to cell numbers by measuring the DNA per sample and comparing this determination with the DNA content of a known number of euploid human cells. Applying this method to our present material we estimate that our patch size within the myometrium is in the order of seve al

thousand cells and possibly as high as 10,000 cells. Assuming a patch of 10,000 cells with a regular (cubic) shape, we can estimate the chance that a tumor arises from a given number of adjacent cells of like phenotype. The probability that a single tumor can arise from two like cells is equal to the ratio of like pairs of adjacent cells to the total number of pairs of adjacent cells. This turns out to be 0.96 and the corresponding probability of our 121 runs is less than 5 per cent. We can thus conclude that if no selection is at play during the growth of leiomyomas, our data rules out the possibility that the tumors arise from two cells of like phenotype.

Although the total number of A and B tumors was about equal, the number of A and B tumors in any particular uterus varied significantly from the expected ratio of 1:1. The distribution appears bimodal or simply skewed. In 10 of 12 cases, the predominant cell in the myometrium in each uterus was the same as the predominant tumor type within that uterus. The deviation in the myometrium was not so marked as in the tumors but the difference is not significant. It is also interesting to note that the $A:B$ distribution in all of the uteri tended toward a more bell shaped curve than the $A:B$ ratio of cells in the tumor bearing uterus (figure 2) but the difference here is also not significant and so unfortunately we do not know the $A:B$ distribution in uteri containing multiple tumors.

The presence of a skewed distribution of A and B tumors within any particular uterus could be due to (1) selective forces limited to the tumors, (2) a bimodal distribution in the $A:B$ ratio of the myometrium, or (3) it could be that selective forces act upon both the myometrium and the tumors.

If tumors arose from a single cell from a random population of cells within the myometrium one would expect that the relative number of A and B tumors within any single uterus would be the same as the $A:B$ ratio of cells within the myometrium. With one exception in ten cases which are suitable for analysis, this seems to be true and the relative number of A and B tumors in a particular uterus tend to be the same as the $A:B$ ratio within the uterus. The variation in the $A:B$ ratio in the myometrium however is not as great. This may be due to (1) the greater number of supporting cells in the myometrium, (2) insensitivity of our technique to ascertain A and B cells within a mixed population of cells, or (3) random sampling variations. In any case, the variation of A and B tumors within any particular uterus could be due to random selection of a cell in uteri which have an uneven distribution of $A:B$ cells.

If a tumor arose from both A and B cells but selection favored a single cell type, one would expect that some of the uteri would contain tumors of only one (the favored) phenotype; but this is not the case. Instead we find that all of the uteri contain both A and B tumors. These findings are also consistent with the hypothesis that the tumors arose at random from a single cell within an unequal number of A and B cells.

A different but possibly pertinent kind of mosaicism is seen in the immunoglobulin κ and λ antigens found in normal individuals and those with multiple myeloma [6]. Normal individuals have been shown by selective fluorescent

staining of lymph nodes and spleen cells (exclusive of those in and adjacent to lymph follicles) to contain two cell types, one which contains the κ antigen and the other the λ antigen [15]. The κ and λ antigen genetic loci are allelic and in normal individuals, the ratio of κ to λ antigen is about 2:1. Individuals with multiple myeloma have either the κ or the λ antigen but not both and the ratio of the number of individuals with the κ and λ antigen is also 2:1. The conclusion from these studies is that selective forces determine an optimal κ:λ ratio in normal people and from this nonrandom population of cells a random number of κ and λ tumors emerge. In our studies the total number of A and B tumors was about the same so that one would have to assume that selection occurred at another locus on the X chromosome.

What could cause an uneven distribution of the $A:B$ ratio within the myometrium of the multiple tumor bearing uteri (and a more bell shaped curve in all the uteri studied)? One possibility is that we are dealing with a sampling error. More cases are needed to determine the $A:B$ ratio of cells in uteri containing few or no tumors and those containing multiple tumors to see if these preliminary findings are significant. In the event that they are, the findings might indicate that AB uteri with an equal number of A and B cells are less likely to give rise to leiomyomas than those uteri whose $A:B$ ratio deviate significantly from 1:1.

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